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Comparison of Fluorescent-Antibody, Neutralizing-Antibody, and Complement-Enhanced Neutralizing-Antibody Assays for Detection of Serum Antibody to Respiratory Syncytial Virus

TEJ N. KAUL,* ROBERT C. WELLIVER, AND PEARAY L. OGRA

Departments of Pediatrics and Microbiology, State University of New York at Buffalo, and Division of Infectious Diseases, Children's Hospital of Buffalo, Buffalo, New York 14222

Received 15 October 1980/Accepted 26 January 1981

A comparison of three assays for the detection of serum antibody to respiratory syncytial virus (RSV) was carried out on 47 serum samples obtained sequentially from infants and young children with RSV infection. Neutralizing-antibody (NA) activity was determined by a semimicromethod of plaque reduction. Complement-enhanced NA activity was determined by the addition of guinea pig complement to NA assays. RSV antibody responses in immunoglobulin G, immunoglobulin M, and immunoglobulin A classes were determined by using indirect immunofluorescence techniques for the fluorescent-antibody (FAb) assay. Antibody to RSV was detectable by all three techniques as early as 4 days after the onset of illness. At all phases of illness, titers obtained by complement-enhanced NA assays were significantly greater than those obtained by NA or FAb assays ($P < 0.01$). RSV-FAb titers determined in the immunoglobulin G class correlated well with those determined by complement-enhanced NA or NA assays. The data suggest that the FAb assay for detection of RSV antibody in serum is somewhat less sensitive but also less laborious and more rapid than NA assays.

Serological studies of respiratory syncytial virus (RSV) infection are hindered by the fact that antibody responses after RSV infection are diminished in infants less than 6 months of age (3), the age group in which severe illness due to RSV occurs most commonly (17). The neutralizing-antibody (NA) assay is the most accurate method currently used for detection of serum antibody to RSV but lacks sensitivity in infants less than 6 months of age with RSV infection (3) and is too laborious for testing large numbers of specimens. Recently, we reported our experiences with indirect immunofluorescence techniques for the fluorescent-antibody (FAb) assay for the detection of RSV antibody in immunoglobulin G (IgG), IgM, and IgA classes in both serum (20) and nasopharyngeal secretions (R. C. Welliver, *Proceedings of the 85th Ross Conference on Pediatric Research: Mucosal and Immune System in Health and Disease*, in press). Other studies have demonstrated the appearance of NA activity which could be enhanced by the addition of fresh guinea pig complement (C') in the serum of patients recovering from infection with several different viral agents (7, 8, 14, 18, 19, 21). Potash et al. (18) reported that neutralizing activity in fresh, unheated serum samples collected from subjects immunized with inactivated RSV vaccine could be markedly reduced by heat inactivation of the samples. The neutralizing activity could be restored by addition of fresh, nonimmune guinea pig or rabbit serum.

The current study was undertaken to determine whether addition of C' enhances neutralizing activity in the serum of patients with natural RSV infection. Moreover, an attempt was made to learn if the results of the FAb assay for the detection of RSV antibody correlate well with those of the NA or complement-enhanced NA (CENA) assays.

MATERIALS AND METHODS

Study group. Forty-seven serum specimens were drawn, at various intervals after the onset of primary RSV infection, from children who varied in age from 1 to 12 months. The patients were diagnosed as having upper respiratory illness only, pneumonia, bronchiolitis, or asthma. All patients were enrolled in a separate ongoing study of respiratory illness in childhood. All children were carefully monitored and were therefore known to be undergoing primary infection with RSV. At the time of initial enrollment, parents of the participants were informed of the goals and risks of the study and of the fact that specimens obtained might be used later for other studies of the immune response to RSV. A signed statement of informed consent was obtained at that time. RSV infection was confirmed

by identification of viral antigen in nasopharyngeal secretions by indirect immunofluorescence techniques (11) and later by recovery of virus by tissue culture infectivity.

FAb test. FAb titers were determined in the IgG, IgM, and IgA classes as previously described (6). Briefly, monolayers of HEp-2 cells were infected with a stock strain of RSV and fixed with acetone to glass slides. Cells were incubated with serial twofold dilutions of serum for 30 min. After washing, slides were labeled with fluorescein-conjugated goat anti-human IgG, IgM, or IgA globulin (Meloy, Springfield, Va.). Slides were washed and examined by fluorescence microscopy. Controls consisted of uninfected HEp-2 cells and slides incubated with saline instead of serum. FAb assays under these conditions did not result in the appearance of fluorescence in the controls.

Neutralization tests. NA assays were carried out with minor modification of a semimicromethod of plaque reduction described previously (8, 13). Briefly, 2×10^5 HEp-2 cells were seeded into each well of plastic tissue culture plates (Linbro, New Haven, Conn.) and incubated in a humid CO_2 incubator overnight. Heat-inactivated serum was diluted twofold in growth medium in duplicate. Each 0.1 ml of diluted serum was incubated with an equal volume of stock virus diluted to contain 100 to 150 plaque-forming units per ml. C′ (GIBCO Laboratories, Grand Island, N.Y.), which had been titrated and diluted to contain 8 U/0.1 ml, was added to one of the pairs of serum dilutions, and 0.1 ml of balanced salt solution was added to the other serum dilution as a control. Serum-virus mixtures with and without C′ were incubated at room temperature for 1 h, after which 0.1 ml of each mixture was adsorbed onto duplicate wells of tissue culture cell monolayers. Plates were then incubated at 37°C for 2 h before removal of the inocula and rinsing of cells with phosphate-buffered saline. Cell layers were overlayed with 1 ml of 1% agar in medium with 2.5% chicken serum (GIBCO). Plates were sealed and incubated at 37°C for 72 h, after which an additional overlay was added. Plates were incubated 4 more days and then fixed in 10% Formalin. After removal of the agar, the cells were stained with 1% crystal violet. NA and CENA titers were taken as the serum dilutions at which a 60% reduction in plaque-forming units was observed.

Controls consisted of virus diluted in medium without serum, virus with C′ without serum, and serum-virus mixtures to which heated C′ was added. Addition of heated C′ did not result in an increase in titers as determined by standard NA assays. The incubation of C′ and virus without serum did not result in a reduction in plaque-forming units.

Statistical analysis. Regression lines were drawn by the method of least squares, and the coefficients of correlation (r values) were calculated to determine the degree of statistical significance.

RESULTS

Kinetics of FAb, NA, and CENA responses. A comparison of FAb, NA, and CENA responses is shown in Fig. 1. Each point on the individual curves represents the geometric mean

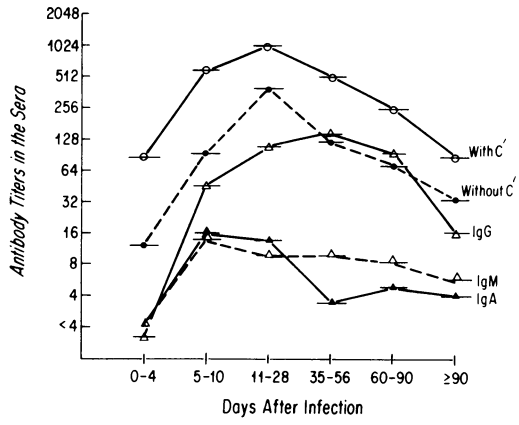

FIG. 1. *Kinetics of serum antibody response to RSV as determined by IgG-specific FAb (△——△), IgA-specific FAb (▲), IgM-specific FAb (△- - -△), NA (●), and CENA (○) assays. Each point represents the geometric mean titer (indicated by cross bars) of at least six samples.*

(indicated by cross bars) of titers obtained from at least six samples. RSV antibody, as determined by the FAb assay with IgG, was present at a geometric mean titer of 2.8 in the first 4 days of illness. An increase in the IgG-specific FAb (IgG-FAb) titer was evident by 5 to 10 days after the onset of illness, and peak titers (111 to 138) were attained between 11 and 56 days after the onset of illness. NA titers were two- to fourfold greater than IgG-FAb titers during the first 28 days of illness, after which titers obtained by the NA assay or by the FAb assay for IgG were virtually identical. CENA titers were 2- to 6-fold and 4- to 30-fold greater than NA and IgG-FAb titers, respectively, at all phases after the onset of illness. CENA titers reached a peak geometric mean titer of 1,023 at 11 to 28 days postillness and decreased to a geometric mean value of 99 by 90 days postillness. Enhancement of NA by C′ was most prominent in the first 10 days of illness, at which time CENA titers were sixfold greater than NA titers. From 35 days postillness until the end of the follow-up period, CENA titers exceeded NA and IgG-FAb titers by two to fourfold. The differences between mean CENA titers and NA or FAb titers were statistically significant at all intervals, as determined by the Student t test ($P < 0.01$).

Comparison of CENA, NA, and FAb titers at each serum dilution. Titers of RSV antibody obtained by each neutralization technique are plotted against titers obtained by FAb assays in the three immunoglobulin classes at each serum dilution in Fig. 2 and 3. Lines of identity are superimposed to emphasize that titers ob-

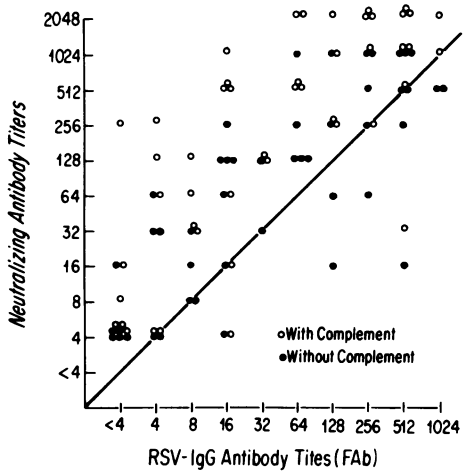

FIG. 2. *Comparison of titers obtained with IgG-specific FAb assays for RSV antibody with those obtained with NA (●) and CENA (○) assays. Line of identity is superimposed.*

tained by neutralization assays were almost uniformly higher than those obtained by FAb assays for RSV-IgG, RSV-IgM, and RSV-IgA at each serum dilution tested.

Correlation of titers obtained by complement-enhanced neutralization with IgG-, IgM-, and IgA-FAb titers. Regression lines indicating the correlation of RSV-FAb titers in each immunoglobulin class tested with titers obtained by complement-enhanced neutralization are shown in Fig. 4. Since the plotting of serum dilutions as shown in Fig. 2 and 3 resulted in an exponential scale which was inappropriate for the plotting of regression lines, the scale in Fig. 4 is a linear one in which each increment on the ordinate and abscissa represents a numerically equal increase in antibody titer. Slopes of the regression lines indicate that the NA assay results correlated best with FAb assay results for RSV-IgG, less well for RSV-IgM, and least for RSV-IgA. However, calculation of correlation coefficients for each regression line indicated a statistically significant (each r value, ≥ 0.474; each P value, < 0.001) correlation of FAb titers in each immunoglobulin class with titers obtained by complement-enhanced neutralization.

Correlation of CENA, NA, and IgG-FAb titers in seronegative to low-titer specimens. In the present study, no serum specimens were seronegative for antibody when tested by NA or CENA assays. The serum samples of three patients with RSV infection (patients 1 through 3, Table 1) were seronegative by the IgG-FAb assay and had low titers of NA. Addition of C′ increased the detectable NA titer 2- to 16-fold. In contrast, the serum samples of four patients known to be undergoing their first episode of respiratory illness and known to be infected with a virus other than RSV (patients 4 through 7, Table 1) were also seronegative for RSV by the IgG-FAb assay and had low titers of NA. Addition of C′ did not result in an increase in the antibody titer in these patients.

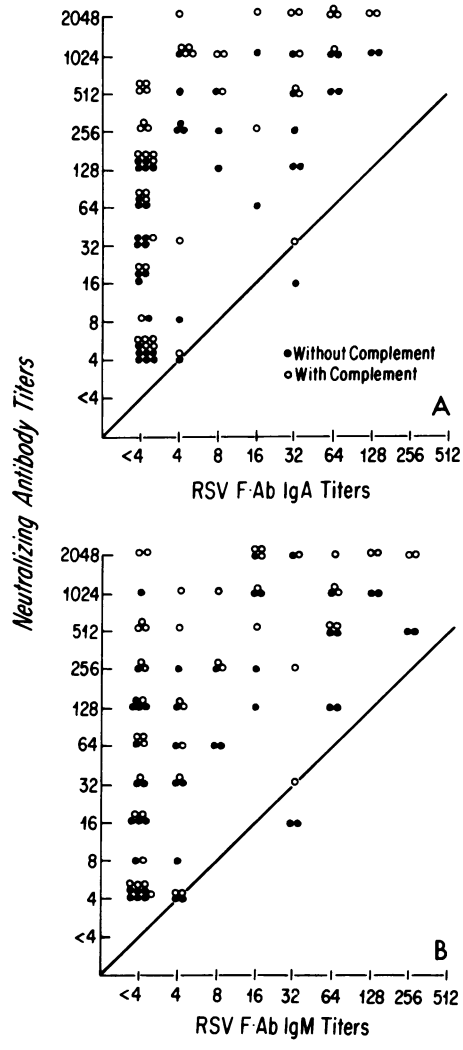

FIG. 3. *Comparison of titers obtained with IgA-specific (A) and IgM-specific (B) FAb assays for RSV antibody with those obtained with NA (●) and CENA (○) assays. Lines of identity are superimposed.*

DISCUSSION

The FAb assay has been used in our laboratory (20; R. C. Welliver, in press) and by other researchers (15) for the detection of RSV anti-

TABLE 1. *NA and CENA titers in serum specimens seronegative for RSV IgG-FAb antibody*

Patient no.	FAb titer in IgG class	NA titer	CENA titer	Fold increase in NA titer by addition of C′	Nature of disease
1	<4	4	8	2	Primary RSV infection
2	<4	4	16	4	Primary RSV infection
3	<4	16	256	16	Primary RSV infection
4	<4	4	4	0	Non-RSV infection
5	<4	4	4	0	Non-RSV infection
6	<4	4	4	0	Non-RSV infection
7	<4	4	4	0	Non-RSV infection

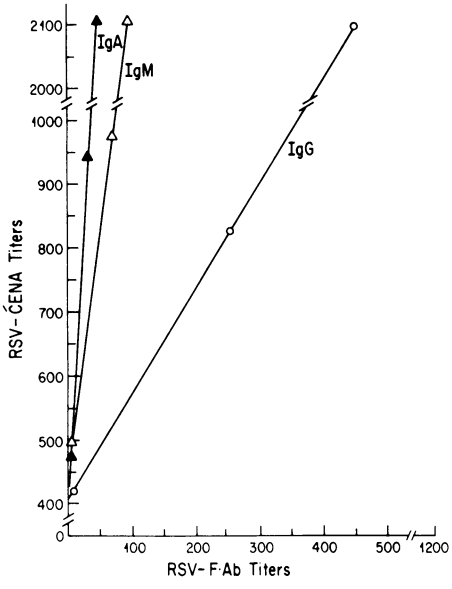

FIG. 4. *Regression lines drawn to show the correlation of CENA titers with FAb titers in various immunoglobulin classes. Symbols: ○, IgG-FAb titers; △, IgM-FAb titers; and ▲, IgA-FAb titers. Each r value, ≥0.474, each P value, <0.01. The ordinate and abscissa scale is linearly adjusted so that each increment represents a numerically equal increase in serum dilution.*

body in serum and secretions. McIntosh and associates (15) studied 19 serum specimens and found an excellent correlation between antibody titers determined by NA assays (without added C′) and by IgG-FAb assays. However, the results of NA assays for RSV antibody in secretions did not correlate well with the results of IgA-FAb assays for the detection of secretory antibody. We undertook the current study to determine the correlation of titers of RSV antibody in IgG, IgM, and IgA classes as measured by the FAb assay with the titers obtained by NA assays with and without added C′ at various intervals after natural infection. This study demonstrated a good linear correlation ($P < 0.001$) of the results of IgG-FAb assays with those of NA and CENA assays. The correlation of results of IgM-FAb or IgA-FAb assays with those of NA and CENA assays was less direct, but still statistically significant. In fact, the use of IgG-FAb assays resulted in detection of titers which were identical to those determined by NA assays in samples taken after the first month of illness. We believe that titers of NA observed in the first month of illness were greater than IgG-FAb titers because of the contribution of NA in the IgM class. Although we could not confirm it by separation of the IgM fraction of our samples on sucrose gradients because of limited sample volume, other studies (9) have shown that the 19S fraction of antibody developing in rabbits after vaccination with herpes simplex virus contributes to the total amount of specific NA formed. The CENA assay appeared to be more sensitive than the IgG-FAb assay for the detection of RSV antibody. Nevertheless, the results of IgG-FAb assays correlated well with those of CENA assays; also, the FAb assay was technically simpler to perform, and results of FAb assays were available roughly 7 days earlier than those of NA or CENA assays. In addition, the FAb assay can be used for the detection of RSV-IgM antibody and therefore may be useful in the early serological diagnosis of RSV infection (20). Finally, the ability to detect RSV antibody in the 11S IgA class (15; R. C. Welliver, in press) makes this technique useful in the study of the secretory antibody response to RSV infection. The apparent presence of non-antibody neutralizing substances in secretions (15) may prohibit the use of NA or CENA assays for the study of RSV secretory antibody responses.

The current study also confirms previous reports that the addition of complement to standard NA assays markedly increases the sensitivity of such assays for the detection of antibody to RSV (1, 2, 18) as well as many other viruses (7, 8, 14, 19, 21). Potash and associates (18) found that heating convalescent serum from recipients of RSV vaccine for 30 min at 56°C reduced the titer of NA and that the neutralizing activity could be restored by the addition of unheated

rabbit or guinea pig serum. However, the number of sera tested was not specified in the report. Buynak et al. (2) compared the results of NA assays with and without the addition of fresh C′ in nine convalescent serum samples also obtained from RSV vaccine recipients. The authors stated that the results of these nine selected specimens reflected the results of a more extended study. Enhancement of antibody titer after the addition of C′ ranged from 2- to 32-fold. Finally, Baughman and associates (1) added C′ to pooled ferret, guinea pig, and horse sera. NA activity of ferret and guinea pig (but not horse) sera was enhanced by the addition of C′. The current study demonstrated that, at all intervals after natural infection, CENA titers were two- to sixfold greater than NA titers. Enhancement of NA activity by C′ was most pronounced in the first 10 days after the onset of illness. In a similar fashion, Takabayashi and McIntosh (19) found that C′ enhancement of NA activity against vaccinia virus was greater in sera obtained 4 weeks after vaccination than in sera obtained 6 months after vaccination, whereas Hampar et al. (9) showed that sera obtained shortly after immunization of rabbits with herpesvirus demonstrated NA activity which could be enhanced by the addition of C′, although NA activity in sera obtained later could be enhanced to a much lesser degree by the addition of C′. The reason that addition of C′ has such a variable effect on NA activity in sera drawn at various intervals after infection is unknown. Hampar et al. (9) speculated that different subclasses of IgG antibody, with differing propensities for fixation of C′, were present at various intervals after antigenic exposure.

In the current study, serum samples obtained from four patients (4 through 7, Table 1) known to have no previous infection with RSV had NA and CENA titers of 4. Since these patients were young infants, the observed titers probably represented maternal antibody acquired transplacentally several months earlier. Antibody titers in these four patients were not enhanced by the addition of C′. The results obtained in this small number of patients suggest that the addition of C′ to NA assays may be a useful test for distinguishing NA which is present as a result of infection from that which is acquired transplacentally.

Dulbecco and associates (5) demonstrated that incubation of Western equine encephalitis virus or poliovirus with specific neutralizing antisera did not totally inactivate the virus even when high concentrations of antisera were used. The results of their studies indicated that virus remained active and infectious in the form of virus-antibody complexes. Addition of fresh rabbit serum to the virus-antibody mixture generally resulted in a complete reduction in the titer of infectious virus. The authors suggested that the addition of fresh serum supplied certain cofactors necessary for complete neutralization. The results of the current study and others suggest that the factor supplied by fresh guinea pig serum was probably C′.

The persistence of infectious virus antibody complexes in vitro, as well as the kinetics of formation and dissociation of these complexes, have been reviewed previously (14). In vivo, infectious herpes simplex virus-antibody complexes have been demonstrated in fluid obtained from vesicular lesions of humans with recurrent herpes labialis (4). Poliovirus-antibody complexes have been recovered from human feces from which virus could be eluted by incubation of complexes at low pH (12). The recovery of RSV from secretions containing specific antibody to the virus has been suggested as demonstrating the existence of virus-antibody complexes in secretions obtained after RSV infection (15). It is well known that infection with RSV occurs commonly despite the presence of transplacentally-acquired NA or serum and secretory antibody acquired by previous infection (10, 16). Failure to fix complement to such virus-antibody complexes, if they occur in vivo, may be a potential mechanism by which reinfections with RSV occur despite the presence of preexisting neutralizing serum or secretory antibody.

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LITERATURE CITED

1. **Baughman, R. H., J. D. Feneters, G. S. Marquis, and J. C. Holper.** 1968. Effect of complement and viral filtration on the neutralization of respiratory syncytial virus. Appl. Microbiol. **16**:1076–1080.
2. **Buynak, E. B., R. E. Weibel, A. J. Carlson, A. A. Mclean, and M. R. Hilleman.** 1979. Further investigation of live respiratory syncytial virus vaccine administered parenterally. Proc. Soc. Exp. Biol. Med. **160**: 272–277.
3. **Chanock, R. M., H. W. Kim, and A. J. Vargosko.** 1961. Respiratory syncytial virus: virus recovery and other observations during 1960 outbreak of bronchiolitis, pneumonia, and minor respiratory diseases in children. J. Am. Med. Assoc. **176**:647–653.
4. **Daniels, C. A., S. G. LeGoff, and A. L. Notkins.** 1975. Shedding of infectious virus/antibody complexes from vesicular lesions of patients with recurrent herpes labialis. Lancet **ii**:524–528.
5. **Dulbecco, R., M. Vogt, and A. G. R. Strickland.** 1956. A study of the basic aspects of neutralization of two

animal viruses, Western equine encephalitis virus and poliomyelitis virus. Virology **2**:162-205.

6. **Gardner, P. S., and J. McQuillin (ed.).** 1974. Rapid virus diagnosis. Application of immunofluorescence, p. 103-117. Butterworths, London.
7. **Graham, B. J., Y. Minamishima, G. R. Dressman, H. G. Haines, and M. Benyish-Melnick.** 1971. Complement-requiring neutralization antibodies in hyperimmune sera to human cytomegalovirus. J. Immunol. **107**: 1618-1630.
8. **Grose, C., J. B. Edmond, and P. A. Brunell.** 1979. Complement enhanced neutralizing antibody response to varicella-zoster virus. J. Infect. Dis. **193**:432-437.
9. **Hampar, B., A. L. Notkins, M. Mage, and M. A. Keehan.** 1968. Heterogeneity in the properties of 7S and 19S rabbit neutralizing antibodies to herpes simplex virus. J. Immunol. **100**:586-593.
10. **Johnson, K. M., H. H. Bloom, M. A. Mufson, and R. M. Chanock.** 1962. Natural reinfection of adults by respiratory syncytial virus: possible relation to mild upper respiratory disease. N. Engl. J. Med. **267**:68-72.
11. **Kaul, A., R. Scott, M. Gallagher, M. Scott, J. Clement, and P. L. Ogra.** 1978. Respiratory syncytial virus infection: rapid diagnosis by use of indirect immunofluorescence. Am. J. Dis. Child. **132**:1088-1090.
12. **Keller, R., and J. E. Dwyer.** 1969. Neutralization of poliovirus by IgA coproantibodies. J. Immunol. **101**: 192-202.
13. **Kisch, A. L., and K. M. Johnson.** 1969. A plaque assay for respiratory syncytial virus. Proc. Soc. Exp. Biol. Med. **112**:583-589.
14. **Majer, M.** 1972. Virus sensitization. Curr. Top. Microbiol. Immunol. **58**:69-84.
15. **McIntosh, K., H. B. Masters, I. Orr, R. Chao, and R. M. Baskin.** 1978. The immunologic response to infection with respiratory syncytial virus in infants. J. Infect. Dis. **138**:24-32.
16. **Mills, J., J. E. Vankirk, P. F. Weight, and R. M. Chanock.** 1971. Experimental respiratory syncytial virus infection of adults: possible mechanisms of resistance to infection and illness. J. Immunol. **107**:123-130.
17. **Parrott, R. H., H. W. Kim, J. O. Arrobio, D. S. Hodes, B. R. Murphy, C. D. Brandt, E. Camargo, and R. M. Chanock.** 1973. Epidemiology of respiratory syncytial virus infection in Washington, D.C. Am. J. Epidemiol. **98**:289-300.
18. **Potash, L., R. S. Lee, and B. M. Sweet.** 1966. Increased sensitivity for detection of respiratory syncytial virus neutralizing antibody by use of unheated sera. Bacteriol. Proc., p. 122.
19. **Takabayashi, K., and K. McIntosh.** 1973. Effect of heat-labile factors on the neutralization of vaccinia virus by human sera. Infect. Immun. **8**:582-589.
20. **Welliver, R. C., T. N. Kaul, T. I. Putnam, M. Sun, K. Riddlesberger, and P. L. Ogra.** 1980. The antibody response to primary and secondary infection with respiratory syncytial virus: kinetics of class specific responses. J. Pediatr. **96**:808-813.
21. **Yoshino, K., and S. Taniguchi.** 1966. Evaluation of the demonstration of complement-requiring neutralizing antibody as a means for early diagnosis of herpes virus infection. J. Immunol. **96**:196-203.